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# Decreased Tumorigenicity of a Human Colon Adenocarcinoma Cell Line by an Antisense Expression Vector Specific for c-*Src*[1]

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## Abstract

In greater than 80% of colon tumors and established cell lines, the specific activities of the protein tyrosine kinases pp60$^{c-src}$ and pp62$^{c-yes}$ are increased with respect to normal colonic epithelial cells. However, no mutations in either gene have been identified in colon tumors. Therefore, the possible biological consequences of activations of these protein tyrosine kinases in colon tumors have been unclear. To determine if pp60$^{c-src}$ activation affects growth and tumorigenicity of established colon tumor cell lines, an antisense expression vector that specifically reduces pp60$^{c-src}$ expression was constructed. The vector was transfected into HT 29 cells, an established colon tumor cell line in which both pp60$^{c-src}$ and pp62$^{c-yes}$ are activated. Two stable subclones were isolated in which pp60$^{c-src}$ but not pp62$^{c-yes}$ expression and activity were reduced. These established cell lines proliferated more slowly than parental cells proportionately to reduction in pp60$^{c-src}$ expression. When injected into nude mice, antisense transfected cells formed slow-growing tumors; however, the rate of tumor growth was reduced far greater than would be predicted from decreased proliferation rates in tissue culture. In contrast, stable subclones transfected with a comparable "sense" expression vector were unaltered in growth rates in tissue culture and in nude mice with respect to parental HT 29 cells. These data demonstrate that the activation of pp60$^{c-src}$ alone contributes to the tumorigenicity of HT 29 cells, a cell line widely used as a model for biological properties of colon carcinoma. Furthermore, because pp60$^{c-src}$ and pp62$^{c-yes}$ appear redundant to the growth regulation of normal colonic epithelial cells, the data suggest that src-specific inhibitors might be of therapeutic value for colon cancer.

## Introduction

In the past few years, significant advances have been made toward understanding the molecular basis for the development of colorectal cancer. In both sporadic and familial colon cancers, mutations in specific oncogenes, such as c-*ras*, in tumor suppressor genes, such as *APC*, *MCC*, and *p53*, and in DNA repair genes, such as *MSH 2*, have all been implicated as important to the development of a majority of tumors (1). However, considerably less is known about the cumulative effects of mutations in these genes on growth-regulatory signal transduction pathways of colonic epithelial cells. One family of signal transduction enzymes, the nonreceptor PTKs[4] of which *src* is the prototype, is activated in nearly every human colon tumor, resulting in specific activities of the enzymes equivalent to the cognate retroviral oncogene products (2–4). For pp60$^{c-src}$, activation is first observed in an early stage of tumorigenesis, beginning with polyps of high malignant potential (5) and persisting through subsequent stages of tumor progression. Furthermore, additional increases in enzymatic activity occur during the development of distant metastases (4–6). Similar activations of pp62$^{c-yes}$ have been observed in colonic polyps and primary tumors (7, 8). Sporadic activation of p62$^{c-lck}$ has also been reported in some colon tumor cell lines (9).

The mechanism for the observed alterations of enzymatic activity of *src* family PTKs in colon tumors is unknown but would appear to result from altered posttranslational regulation of the PTK activity, because mutations in the *src* family genes are not found in colon tumors (10). Several growth-regulatory pathways in which pp60$^{c-src}$ kinase activity is increased have been identified. For example, in fibroblasts, regulatory signals that increase the PTK activity of *src* family kinases include aggregation of integrins (11) and possibly other extracellular matrix components and the addition of several mitogenic peptides (12). Additionally in the cell cycle, *src* family activity is required in $G_2$ phase for fibroblast cell division (13) and is activated during M phase (14). Thus, activation of *src* family PTKs during tumorigenesis of colonic epithelial cells may be the result of primary genetic changes in genes other than c-*src* that lead to constitutive deregulation of one of the pathways in which c-*src* participates.

Regulation of *src* family PTKs appears to be important in normal colonic epithelial cell growth and differentiation. Normal colonic epithelial cells express both pp60$^{c-src}$ and pp62$^{c-yes}$ (15), and the activity of these enzymes is higher in actively dividing crypt cells than in more differentiated cells (16). Thus, high specific activity of *src* family tyrosine kinases may be a marker of proliferation. However, hyperproliferation

Received 10/9/96; revised 12/20/96; accepted 12/23/96.
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[4] The abbreviation used is: PTK, protein tyrosine kinase.

alone is insufficient to explain increased pp60$^{c\text{-}src}$ activity in malignant and premalignant colon epithelial cells, because the enzymatic activity is increased in ulcerative colitis as opposed to inflammation (17) and in polyps of high malignant potential as opposed to more "benign" polyps (5). Activity remains high in primary tumor and in metastases. Additionally, PTK inhibitors greatly reduce the growth of colon tumor cell lines with activated pp60$^{c\text{-}src}$ (18). These results suggest that *src* family activation may contribute to the altered growth regulation of colon tumor cells. Whether these changes are important to tumorigenesis of colonic epithelial cells or are only the result of malignant transformation was examined in this study by specifically inhibiting the expression of pp60$^{c\text{-}src}$ in a well-characterized human colon adenocarcinoma cell line, HT 29.

## Results

To accomplish inhibition of pp60$^{c\text{-}src}$ expression, we used antisense oligonucleotides and expression vectors directed against the translation start site of pp60$^{c\text{-}src}$. As a model cell line, HT 29 human colon adenocarcinoma was used because both pp60$^{c\text{-}src}$ and pp62$^{c\text{-}yes}$ have high specific activities in these cells. Twenty-two base sense and antisense unmodified oligonucleotides were synthesized, beginning five bases upstream of the ATG translation start site of c-*src*. This region has only 43% homology with c-*yes*. Increasing concentrations of each purified oligonucleotide were added to HT 29 human colon adenocarcinoma cell cultures in serum-free medium. After a 16-h incubation, the cells were harvested and lysed, and kinase activity and protein levels were determined. As shown in Fig. 1, dose-dependent decreases in pp60$^{c\text{-}src}$ kinase level (*A*) and kinase activity (*B*) were observed in HT 29 cells treated with antisense oligonucleotides to a maximum concentration of 75 $\mu$M. No further inhibition was observed at 100 $\mu$M. No changes in either c-*src* protein level (Fig. 1*A*) or activity (Fig. 1*B*) were observed in the cells treated with the sense oligonucleotide compared to the control HT 29 cells. No inhibition of c-*yes* expression or kinase activity was observed (data not shown). These results demonstrate that the c-*src* antisense oligonucleotide specifically inhibits c-*src* kinase activity and protein expression.

To evaluate potential alterations in proliferation and tumorigenicity resulting from reduction of c-*src* expression and activity, the antisense and sense oligonucleotides were incorporated into a pcDNA I/Neo plasmid vector under the control of the cytomegalovirus promoter and transfected into HT 29 cells; clones were selected in G418. The kinase activities and protein levels of pp60$^{c\text{-}src}$ and pp62$^{c\text{-}yes}$ were determined in antibiotic-resistant clones, as described in "Materials and Methods." Fig. 2 compares the results from parental HT 29 cells (*Lanes 1*), two antisense transfectants termed AS15 (*Lanes 2*) and AS33 (*Lanes 3*), and a "sense" transfectant, termed S20 (*Lanes 4*) for kinase activity by autophosphorylation of pp60$^{c\text{-}src}$ or pp62$^{c\text{-}yes}$ (Fig. 2*A*), phosphorylation of an exogenous substrate, enolase (Fig. 2*B*), as well as relative protein levels by immunoblotting (Fig. 2*C*). No differences in expression or activity of pp60$^{c\text{-}src}$ or pp62$^{c\text{-}yes}$ were observed between parental HT 29 cells and the "sense" transfectant S20. However, clone AS15 was

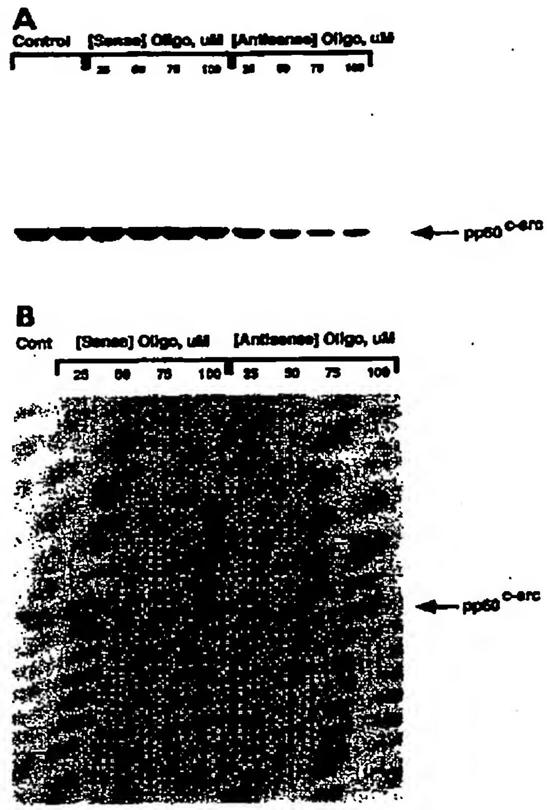


*Fig. 1.* Expression and activity of pp60$^{c\text{-}src}$ in HT 29 colon adenocarcinoma cells treated with c-*src* sense and antisense oligonucleotides. Twenty-two base sense (5'-GGACCATGGGTAGCAACAAGAG-3') and antisense (5'-CTCCTTGTTGCTACCCATGGTCC-3') unmodified oligonucleotides were synthesized as described in "Materials and Methods." Concentrations of each oligonucleotide indicated in the figure were added to subconfluent *HT* 29 cultures (5 × $10^6$ cells) in serum-free medium. After a 16-h incubation, detergent lysates were made in a standard radioimmunoprecipitation assay buffer and subjected to immunoblotting (*A*) or immunoprecipitation and immune complex kinase assay (*B*) for pp60$^{c\text{-}src}$ as described.

reduced 4.5-fold in pp60$^{c\text{-}src}$ activity and level with respect to parental HT 29 cells (compare *Lanes 1* and *2*), and clone AS33 was reduced 2–2.5-fold in pp60$^{c\text{-}src}$ kinase and 2-fold in protein level (compare *Lanes 1* and *3*). To determine the specificity of inhibition of pp60$^{c\text{-}src}$ reduction, the expression and activity of the related *src*-family member pp62$^{c\text{-}yes}$ was assessed. As shown in Fig. 2, the expression and activity of pp62$^{c\text{-}yes}$ in all transfectants were comparable to parental HT 29 cells.

The morphology of AS15 cells in comparison to parental cells is shown in Fig. 3. Subtle morphological differences from parental cells are noted, with AS15 cells growing in a more elongated, organized pattern. Cells at the edge of colonies have a more serrated, villus-like appearance. However, these changes did not correlate with increased expres-

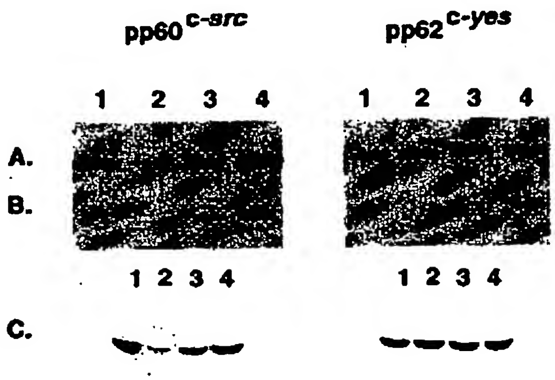


*Fig. 2.* Expression and activity of pp60$^{c\text{-}src}$ and pp62$^{c\text{-}yes}$ in parental HT 29 cells (*Lanes 1*), "antisense" transfected clones AS15 (*Lanes 2*) and AS33 (*Lanes 3*), and a "sense" transfected clone, S20 (*Lanes 4*). Stable HT 29 subclones were isolated, and cell lysates were subjected to immunoblotting and immune complex kinase assays for pp60$^{c\text{-}src}$ and pp62$^{c\text{-}yes}$ as described in "Materials and Methods." *A*, autophosphorylation of pp60$^{c\text{-}src}$ or pp62$^{c\text{-}yes}$; *B*, phosphorylation of the exogenous substrate enolase; *C*, relative levels of pp60$^{c\text{-}src}$ and pp62$^{c\text{-}yes}$.

sion or altered distribution of villin (data not shown). By electron microscopy, the number of microvilli was unaltered, and no evidence for mucin granules was observed. Thus, AS15 cells lack classic hallmarks of differentiation of HT 29 cells. No morphological changes were observed in AS33 cells or in any clones derived from "sense" transfectants.

Proliferation rates of the clonal cell lines in tissue culture are shown in Fig. 4*A*. HT 29 and S20 cells had doubling times of 23 h, as compared to 28 and 36 h for AS33 and AS15 cells, respectively. To examine anchorage-independent growth, soft agar colony formation was assessed as described in "Materials and Methods." No difference in ability to form soft agar colonies was observed between parental HT 29 cells and S20 "sense" transfectants. AS15 cells were 5-fold reduced in number of colonies, and AS33 cells were 2-fold reduced. The number of cells/colony were also fewer for the antisense transfectants; however, longer growth periods did not significantly increase the number of colonies formed by these cells.

To determine if reduced pp60$^{c\text{-}src}$ level and activity affected tumorigenicity and growth in nude mice, two clones, AS15 (antisense transfected), and S20 (sense transfected) were injected in equal numbers ($10^6$ viable cells) into the flanks of nude mice. Growth of tumors induced by these cell lines in nude mice is shown in Fig. 4*B*. In three separate trials of 5, 8, and 8 mice per cell type, 100% of mice inoculated with either parental HT 29 or "sense" transfectants developed tumors. In contrast, only 70% of mice inoculated with the reduced *src* clone, AS15, developed viable tumors. AS15-inoculated mice had a significant lag period in tumor development compared to parental and sense clones, with clearly visible tumors not appearing before 30 days after injection. After 50 days, tumor volume resulting from growth of antisense transfectants was approximately 1000-fold less

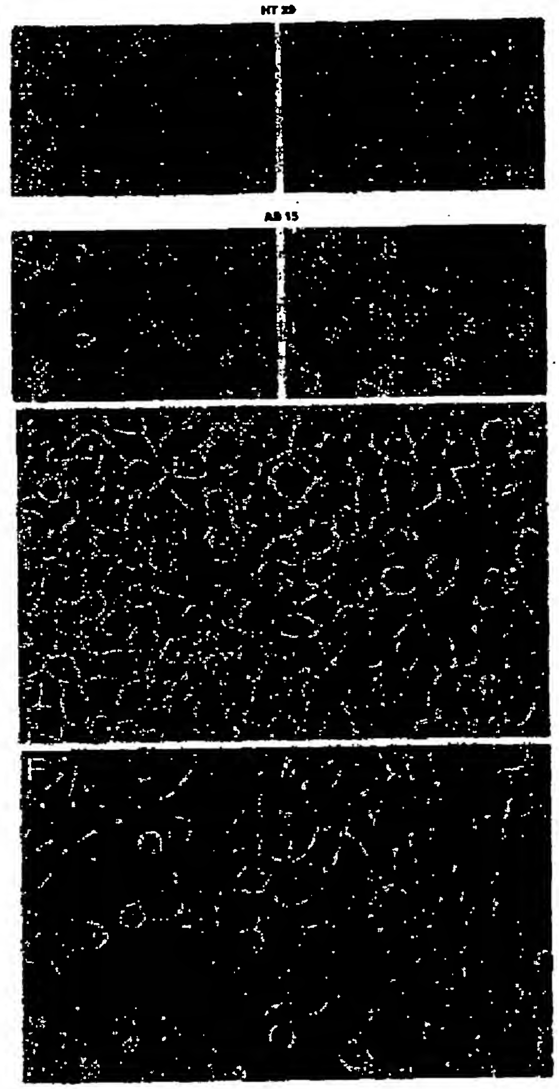


*Fig. 3.* Morphology of AS15 and parental HT 29 cells. Photomicrographs of cell cultures were taken 3 days after seeding $2 \times 10^5$ cells in tissue culture medium. *A* and *C*, ×150; *B* and *D*, ×300. *E*, HT 29, ×400; *F*, AS15, ×400.

than that of the "sense" transfected clone, S20 and parental HT 29 cells. Growth of AS33 tumors was intermediate to that of parental and AS15 clones, in accord with the intermediate steady-state levels of pp60$^{c\text{-}src}$ expressed in this clone. Thus, reduction of pp60$^{c\text{-}src}$ expression corresponds directly with decreased tumorigenicity. Furthermore, the decreased proliferation rate of these in tissue culture is insufficient to account completely for the reduction in growth rate in nude mice.

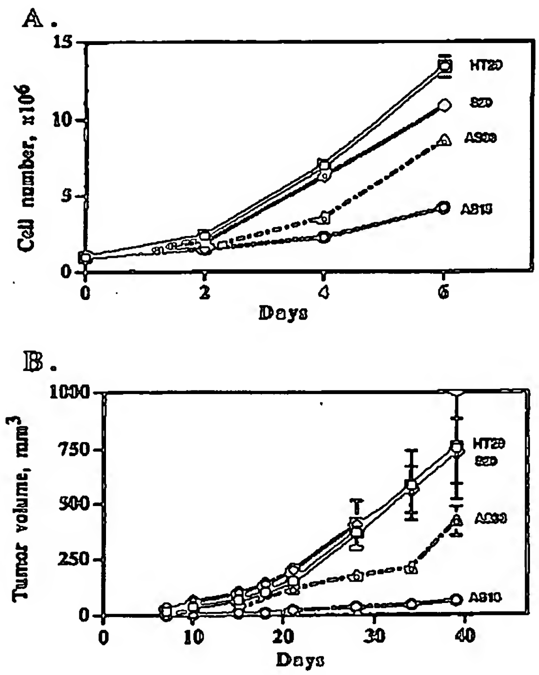


*Fig. 4.* Growth of parental HT 29 cells and clones AS15 and S20 cells *in vitro* and in nude mice. For *in vitro* growth (*A*), 1 × $10^6$ cells were seeded in 60-mm tissue culture dishes with DMEM, supplemented with 10% fetal bovine serum. Viable cells were counted every second day. For *in vivo* growth (*B*), 1 × $10^6$ cells of each clone were injected s.c. in the flank of eight nude mice/cell type. Tumor diameters were measured biweekly with calipers, and tumor volume was calculated. *Bars*, SE.

## Discussion

These findings support an important role for pp60$^{c\text{-}src}$ in the tumorigenicity of a colon adenocarcinoma cell line. Given the frequency of increased specific activity of this PTK in human colon tumors and cell lines, the results suggest that activation of pp60$^{c\text{-}src}$ is a common downstream event following genetic alterations leading to colon cancer. The close association of c-*src* activation with critical early events in malignancy, *i.e.*, activation in polyps of high malignant potential but not in "benign" polyps (5), in ulcerative colitis but not inflammation (17), demonstrates that increased enzymatic activity is likely an important common event in the development of colon cancer. Similar increases in c-*yes* activity have also been observed in early stages of colon tumor development (19, 20). Although the role of c-*yes* activation in early events in colon tumorigenesis is as yet unknown, the results of this study imply that activation of pp60$^{c\text{-}src}$ alone may be critical to sustained tumorigenicity and tumor cell growth.

In two human leukemia cell lines, U937 from a histiocytic lymphoma (21) and K562 from a chronic myelogenous leukemia (22), c-*src* expression has also been implicated as important for proliferation and differentiation by a strategy in which the complete v-*src* gene was expressed in the antisense orientation. These results may suggest a wider role for c-*src* expression in the growth of tumor cells. Alternatively, because the ability of the v-*src* antisense expressed in this vector to inhibit other members of the *src* family was not assessed, inhibition may result from normal functions of the *src* family in growth-regulation pathways.

In the experiments presented in this report, biological effects occurred without c-*yes* inhibition. This result is somewhat surprising, because *src* "knock-out" mice suffer no apparent defects in development or function of the colon (23), suggesting that another family member (presumably c-*yes*) is redundant for c-*src* signaling functions in these processes. However, recent evidence has demonstrated that c-*src* is more important than c-*yes* in the production of vascular endothelial growth factor induced by hypoxia (24). In accord with this result, we have demonstrated that the antisense transfectants are reduced in vascular endothelial growth factor relative to parental cells, proportionately to reduction in steady-state pp60$^{c\text{-}src}$ levels.[5] Thus, decreased vascular endothelial growth factor production might explain the differences in the proliferation rates of antisense transfectants in tissue culture relative to their ability to grow in nude mice. Indeed, mice with AS15-induced tumors have survived for more than 1 year, with tumor burdens no larger than those induced by HT 29 cells in 60 days.

In other model systems in which *src* family activation is required for tumor development, c-*src* and c-*yes* are also not redundant in tumorigenic function. In transgenic mice expressing the polyoma virus-encoded middle T oncogene in mammary epithelium, mammary tumors universally develop (25). When these mice are crossed with "srcless" mice, the progeny do not develop mammary tumors (26). In contrast, when the mice are crossed with mice in which c-*yes* has been functionally deleted, mammary tumors develop consistently, although pp62$^{c\text{-}yes}$, like pp60$^{c\text{-}src}$, is capable of associating with polyoma middle T antigen, an association which increases the specific activities of either *src* family member [reviewed by Courtneidge *et al.* (27)]. Thus, in several tumor systems, activation of pp60$^{c\text{-}src}$ in the absence of mutation may be critical to tumor development. However, to eliminate any potential role for c-*yes* in colon cancer, further experiments will be required.

The mechanism by which increased specific activity of pp60$^{c\text{-}src}$ kinase occurs in colon cancer remains unknown. However, recent data have demonstrated that cytoskeletal and extracellular matrix proteins may induce signaling through *src* [reviewed by Erpel *et al.* (28)]. Because several of the genetic mutations that commonly occur in colon cancer development involve proteins of the cytoskeleton (APC, DCC, and MCC), an obvious possibility is that these proteins play a role in normal regulation of pp60$^{c\text{-}src}$ activity, which is higher in actively dividing crypt cells than in differentiated, nonproliferative colon epithelial cells. The strategy used here, *i.e.*, specific reduction of expression through the use of a small antisense transcript, may provide further insight into the signaling pathways in colon epithelial cells that involve pp60$^{c\text{-}src}$ regulation and the relative roles of *src* family mem-

[5] L. Ellis and G. Gallick, unpublished data.

bers in colon tumorigenesis and progression. Furthermore, the differences between the enzymes may be exploited in the design of PTK inhibitors. A src-specific inhibitor would be expected to have limited toxicity but effectively and specifically reduce the growth rate of colon tumor cells.

## Materials and Methods

**Cell Culture.** HT 29 cells were maintained in DMEM with Earle's salts and 2 mM glutamine (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT). For studies with antisense oligonucleotides, cells were transferred to serum-free media 12 h prior to the addition of the oligonucleotide.

**Immunoprecipitation and Immune Complex Kinase Assays.** Prior to lysis, cells were rinsed twice with ice-cold PBS. Detergent lysates were made in a standard radioimmunoprecipitation assay buffer. Cells were homogenized and clarified by centrifugation at 10,000 × *g*. Cell lysates (250 μg protein) were reacted for 2 h with either monoclonal antibody 327 (Oncogene Sciences) for immunoprecipitation of pp60$^{c\text{-}src}$ or 1B7 (Wako, Inc., Richmond, VA) for immunoprecipitation of pp62$^{c\text{-}yes}$. Immune complexes were formed by the addition of 6 μg of rabbit anti-mouse IgG (Organon Teknika, Durham, NC) for 1 h, followed by 50 μl of 10% (v/v) formalin-fixed Pansorbin (*Staphylococcus aureus*, Cowan strain; Calbiochem, La Jolla, CA) for 30 min. Pellets were washed three times in a buffer consisting of 0.1% Triton X-100, 150 mM NaCl, and 10 mM sodium phosphate. Immune complex kinases assays were performed by standard procedures as described previously (16). Briefly, reactions were initiated at 4°C by the addition to each sample of 10 μCi of [$\gamma$-$^{32}$P]ATP, 10 mM $Mg^{2+}$, and 100 μM sodium orthovanadate in 20 mM HEPES buffer. After 10 min, reactions were terminated by the addition of SDS sample buffer. Proteins were separated by SDS-PAGE on 10% polyacrylamide gels, and radioactive proteins were detected by autoradiography. Quantitation of differences in activity was determined by densitometry.

**Immunoblotting.** Clarified cell lysates (250 μg/lane) were separated by SDS-PAGE on 10% gels and electroblotted onto polyvinylidene difluoride membranes (Amersham Corp., Chicago, IL) using standard procedures (16). Membranes were blocked with 15% skimmed milk in PBS, then incubated with anti-src or anti-yes antibodies at 1:1000 dilution, followed by horseradish peroxidase-conjugated rabbit anti-mouse IgG. Specific binding of antibody was determined using the ECL detection system (Amersham).

**Oligonucleotide Synthesis.** Twenty-two base sense (5'-GGACCATGGGTAGCAACAAGAG3') and antisense (5'-CTCCTTGTTGCTACCCATGGTCC3') unmodified oligonucleotides were synthesized by standard protocols (Millipore Cyclone plus DNA synthesizer), deprotected with 30% $NH_4OH$, and extracted with 1-butanol (29).

**Construction of c-src Expression Vectors and Transfection.** A construct spanning the translation start site of pp60$^{c\text{-}src}$ was generated by annealing two primers (5'-AGCTTGGACCATGGGTAGCAACAAGAGCAAGCCCAAGGAT-3') and (5'-CTAGATCCTTGGGCTTGCTCTTGTTGCTACCCATGGTCCA-3'). The antisense construct was synthesized in a similar manner with the two primers (5'-AGCTATCCTTGGGCTTGCTCTTGTTGCTACCCATGGTCCT-3') and 5'-CTAGAGGACCATGGGTAGCAACAAGAGCAAGCCCAAGGAT-3'). The pcDNAI plasmid (InVitrogen) was then digested with *Hin*dIII and *Xba*I, and a ligation reaction was performed to insert the sense and antisense constructs. *Escherichia coli* was transformed by the plasmids, selected clones were harvested, the bacteria were lysed by alkali treatment, and plasmids were purified. Confirmation of the correct insert was determined by PCR followed by DNA sequencing.

**Transfection.** HT 29 cells were grown to 70% confluency. Cells were transfected in serum-free media for 6 h with the aid of Lipofectamine (Life Sciences) at a ratio of 100 μg of lipofectamine/16 μg of plasmid DNA. G418-resistant colonies were expanded, and the resulting clones were screened for expression and activity of pp60$^{c\text{-}src}$, as described above.

**Tumorigenicity Assays.** Cells from clones to be tested were grown in tissue culture to log phase (~70% confluent), trypsinized, and counted with the aid of a hemocytometer. Cells (1 × $10^6$) of each clone were injected s.c. in the flank of eight nude mice/cell type. After tumors were detected by visual observation, tumor diameters were measured biweekly with calipers. In animals in which tumors formed, the average tumor volume was calculated.

## Acknowledgments

We thank Dr. Hidayuki Saya for advice in constructing the plasmids and Dr. R. B. Arlinghaus for careful reading of the manuscript.

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